REPRODUCTION
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Kinetics of murine decidual dendritic cells

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Abstract

Dendritic cells (DCs) are professional antigen presenting cells (APC) capable of induction of primary immune responses as well as immunologic tolerance. Myeloid and lymphoid subsets of murine DCs are able to shift cytokine responses of T cells toward Th2 and Th1 profiles respectively. Thus, DCs would be suitable candidates to mediate the balance of maternal immune responses to conception. We analyzed pregnancy-related variations in uterus and splenic DCs in a murine model. C57BL/6-mated Balb/c female mice with vaginal plugs were scarified at early, middle, and late pregnancy. Frozen sections of uterus and spleen at each stage of pregnancy were immunostained with CD11c- and MHC-II-specific antibodies. Two-color immunohistochemistry was also carried out using anti-CD11c and one of the antibodies against CD11b, CD8α, CD86, and DEC-205. Using morphometric analysis, the average density of DCs and relative percentage of myeloid ($CD11c^+$, $CD11b^+$) and lymphoid DCs ($CD11c^+$, $CD8a^+$) were determined at each stage. Our results showed that DCs are present throughout the pregnancy in decidua. The average density of decidual DCs at early pregnancy was significantly higher relative to middle and late gestation or to those of endometrial DCs of non-pregnant mice. Interestingly, the average density of decidual and splenic DCs, followed the same variations at different stages of pregnancy. The relative percentage of decidual lymphoid DCs (LDC) was significantly higher at mid-gestation when compared with other stages of pregnancy or non-pregnant mice. Inversely, the frequency of myeloid DCs (MDC) and the MDC/LDC ratio were statistically lower at the middle stage of pregnancy. A majority of decidual DCs expressed MHC-II and CD86. At early pregnancy, DCs were more concentrated subadjacent to the luminal epithelial layers, whereas at mid- or late gestation, DCs were randomly distributed in the stroma and around the epithelium. Mid-pregnancy period was a critical point with regard to splenic DCs kinetics, as both the average density of DCs and the frequency of MDCs decreased significantly when compared with early or late pregnancy, although the relative percentage of splenic LDCs did not change. Our data suggest that the balance of MDC and LDC is finely tuned throughout pregnancy, pointing an eminent immunoregulatory role of DCs in the maintenance of pregnancy.

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Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells (APC), which are essential for initiation of primary immune responses (Steinman 1991, Bancherau *et al.* 2000). These cells serve as the key regulators of immunologic mechanisms and depending on their subsets may induce immunity or immunologic tolerance. DC subsets may provide T cells with the different cytokine/molecule microenvironments that determine the classes of immune response, for example, type 1 versus type 2 CD4 helper cell profiles (Moser & Murphy 2000). At least two distinct pathways of DC development have been identified in mice, myeloid, and lymphoid (Steinman *et al.* 1997, Vremec & Shortman 1997).

Myeloid DCs (MDC) express CD11c and CD11b and induce a vigorous proliferative response in $CD4^+$T cells, whereas lymphoid DCs (LDC) express CD11c, CD8α, and DEC-205 and induce a limited $CD4^+$T cell response

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that is associated with marked T-cell apoptosis (Suss & Shortman 1996, Vremec *et al.* 2000). Both subsets express high levels of class II major histocompatibility complex (MHC-II) and the co-stimulatory molecules, CD86 and CD80 (Banchereau *et al.* 2000).

The mechanisms leading to the fact that semiallogenic fetus is not rejected by the maternal immune system have not been completely understood and in this regard several hypotheses have been proposed. It has been postulated that during pregnancy a local phenomenon of non-specific immunosuppression might take place. Tolerance induction during pregnancy is likely to be related to the unique cocktail of hormones, cytokines, and immunoregulatory cells present at the site of blastocyst implantation (Thellin *et al.* 2000). There are plenty of reports indicating the presence of an immunosuppressive microenvironment at the feto-maternal interface. Fas/FasL induced apoptosis of the immune cells circulating to decidua (Hunt *et al.* 1997, Jerzak *et al.* 1998), presence of complement regulatory proteins at the feto-maternal interface (Holmes *et al.* 1990, 1992, Xu *et al.* 2000), immunoregulatory function of placental indoleamine 2,3 dioxygenase, an enzyme involved in tryptophan catabolism (Kamimura *et al.* 1991, Munn *et al.* 1998), inhibition of natural killer (NK) cell-mediated cytotoxicity by human leukocyte antigen (HLA)-G and HLA-E (McMaster *et al.* 1995, King *et al.* 2000), and pivotal role of regulatory T cells in induction of maternal tolerance to paternal alloantigens (Saito *et al.* 2005, Zenclussen 2005) all are the immunosuppressive mechanisms which are involved in the protection of semiallogenic fetuses. Besides the immunosuppressive mechanisms mentioned above, the cytokine environment during pregnancy is also critical for successful pregnancy. According to several reports, a T-helper (Th)-2 response is necessary for a successful fetal outcome, while a Th1 response results in increased fetal loss (Lin *et al.* 1993, Jenkins *et al.* 2000, Lim *et al.* 2000, Piccinni *et al.* 2000, Croy 2001, Makhseed *et al.* 2001). For example, fetal loss in abortion-prone CBA/j × DBA/2 mating is due to a deficiency in the production of Th2 cytokines (Rajagopalan & Long 1999). In humans, deviation from a Th2 cytokine response leads to spontaneous abortion as well (Hill *et al.* 1995, Raghupathy 2001). Although many attempts have been made to highlight the supporting role of Th2 cytokines, there are also some reports indicating that Th1 cytokines may serve a pivotal role during pregnancy (Svensson *et al.* 2001, Chaouat *et al.* 2002, 2004*a*, 2004*b*). It must be taken into consideration, however, that there are sequential windows of Th1/Th2 dominance and pregnancy is associated with very precise timing and tuning of such cytokines as, for example, Th1 cytokines exert their supporting role during early gestation period (Chaouat *et al.* 2002).

Regarding the importance of Th2 immunity as well as induction of tolerance to fetal allograft during pregnancy, and having considered the mutual role of DCs in induction of immunity versus tolerance and Th1 versus Th2 immune responses, it seems that these cells would be potentially suitable candidates to mediate the balance of maternal immune responses against pathogens, while retaining its capacity to support pregnancy. In fact, there is evidence that DCs could play a protective role at the human feto-maternal interface (Kammerer *et al.* 2000).

In our recent report, we showed variations of murine endometrial DC subsets during estrous cycle (Zarnani *et al.* 2006). In this study, we evaluated the frequency, localization, and immunophenotype, with emphasis on myeloid and lymphoid markers, of murine decidual DCs during early, middle, and late stages of pregnancy. In addition, to address the systemic effect of pregnancy on DC kinetics, the same variables were studied in splenic DC populations.

Materials and Methods

Animals

Female Balb/c mice (8–12 weeks) were prepared from Pasteur Institute of Iran. Mice were kept under optimal conditions of hygiene, temperature, and humidity with 12 h light:12 h darkness cycle and were allowed food and water *ad libitum*. All experimental procedures on animals were approved by the ethical committee of Avesina Research Institute (ARI).

Determination of gestational age

Female Balb/c mice were allowed to mate with a male C57BL/6 and checked daily for the presence of vaginal plug. Females with vaginal plug were separated and examined for the presence of sperm in vaginal smear. The day of vaginal plug and sperm detection was considered to be the day 0.5 of pregnancy.

Tissue specimens

Non-pregnant mice and pregnant mice on gestation days (GD) 2, 11, or 18, corresponding to early, middle, and late pregnancy periods respectively, were killed and the middle one-third of left horn of the uterus as well as spleen were removed simultaneously. Frozen sections of the tissues were cut at 5 μm thickness, transferred to glass slides, air-dried at room temperature for 8 h and fixed in ice-cold acetone for 2 min. The slides were then stored at −70 °C until used.

Antibodies

The primary and secondary antibodies used in this study together with their clone, origin, isotype, and optimal working dilutions are listed in Table 1.

Table 1 Primary and secondary antibodies used for immunohistochemical staining.

Antigen	Clone	Species/ isotype	Working dilutions[a]	Supplier
CD8α	83-6.7	Rat/IgG2a	1:250	Pharmingen
DEC-205	NLDC-145	Rat/IgG2a	1:10	Serotech
CD86	GL1	Rat/IgG2a	1:70	Pharmingen
CD11b	M1/70	Rat/IgG2b	1:250	Pharmingen
CD11c	HL3	Hamster/IgG group 1	1:70	Pharmingen
I-A/I-E	2G9	Rat/IgG2a	1:250	Pharmingen
Rat Ig[b]	–	Goat (multiple-adsorbed)	1:70	Pharmingen
Hamster IgG[c]	G70-204/G94-56	Mouse/IgG1 and IgG2b	1:250	Pharmingen

[a]All dilutions were made with antibody diluent (Pharmingen, Sydney, Australia). [b]HRP-conjugated. [c]Biotin-conjugated.

Immunohistochemistry

Acetone-fixed cryostat sections of uterus and spleen were thawed and washed thrice with 0.15 M TBS (Tris-buffered saline, pH 7.4). The slides were then incubated for 10 min with 5% normal mouse, goat or rat sera appropriately diluted in TBS to block non-specific bindings. For MHC-II staining, the slides were incubated with appropriate dilution of biotin-conjugated rat anti-mouse I-A/I-E for 1 h. Following three washes, endogenous peroxidase activity was quenched by the addition of 0.3% H_2O_2 in TBS. After washing with TBS, slides were incubated with horse radish peroxidase (HRP)-conjugated streptavidin (Biosource International, NY, USA) diluted in a ratio of 1:50 in TBS for 30 min. Excess reagents were washed off by washing thrice with TBS followed by the addition of $NiSO_4$-supplemented diaminobenzidine (DAB; Sigma) as substrate. Sections were counterstained with Harris hematoxylin (Sigma), dehydrated with ascending grades of ethanol each for 30 s and mounted in Entellan (Merck).

Staining with anti-CD11c differed from the above procedure in that the slides were stained with hamster anti-mouse CD11c, followed by incubation with biotin-conjugated mouse anti-hamster IgG for 45 min. The staining was completed as above.

For DEC-205, CD8α, CD11b, and CD86 staining, briefly, sections were sequentially incubated with optimally diluted primary antibodies followed by washing and addition of HRP-conjugated goat anti-rat secondary antibody. The sections were then developed and processed as above.

Two-color immunohistochemistry

Adaptation for immunohistochemical double staining was as follows: briefly, tissue sections were sequentially incubated with appropriate dilutions (Table 1) of hamster anti-mouse CD11c and of rat anti-mouse DEC-205, CD8α, CD11b, or CD86 monoclonal antibodies for 75 min at room temperature followed by a mixture of HRP-conjugated goat anti-rat immunoglobulin and biotin-conjugated mouse anti-hamster IgG, and alkaline phosphatase-conjugated avidin–biotin complex (ABC; DAKO, Glostrup, Denmark). The peroxidase activity was visualized first in a controlled manner under light microscope using DAB as substrate. Following three washes with TBS, the alkaline phosphatase activity was developed by 20–30 min incubation in the alkaline phosphatase blue substrate (Vector Laboratories, Burlingame, CA, USA). Next, the sections were rinsed in tap water, counterstained with nuclear fast red (DAKO, Glostrup, Denmark), dehydrated not more than 30 s in absolute ethanol, cleared with Histoclear (Vector Laboratories), and mounted with Vecta mount (Vector Laboratories). In these preparations, the alkaline phosphatase activity yielded a blue reaction product, whereas peroxidase activity appeared brown. Levamisole (0.024%; Sigma) and H_2O_2 (0.3%) were used to block endogenous alkaline phosphatase and peroxidase activities respectively. For co-localization of CD11c and MHC-II, serial sections of tissues were stained separately with these antibodies. In negative control slides, primary or secondary antibodies were omitted. Species-matched control sera, diluted in a ratio of 1:10, were also used, instead of primary antibodies as isotype-matched Ab and the results were shown to be always negative (Fig. 1d and h).

Morphometry

To evaluate the relative percentage of DCs expressing one of the markers of interest (CD8α, DEC-205, CD11b, and CD86), double positive cells were counted at 400× magnification in 40 individual fields of 0.0625 mm^2 (totally 2.5 mm^2) for each section and expressed as a percentage of the total number of $CD11c^+$ cells. In single-color immunostainings for CD11c, positive cells were also counted in the same manner and expressed as a percentage of the total number of nucleated cells.

Statistical analysis

Five animals were used in each experimental group. Comparisons were made by non-parametric Kruskal–Wallis test. Correlation analysis was performed using Pearson's test. $P<0.05$ was considered statistically significant.

Results

Distribution of MDC and LDC in spleen and decidua

To identify distribution and localization of MDCs and LDCs in spleen and decidua of pregnant mice, a total number of five animals were used in each experimental group. Tissue sections were stained for expression of

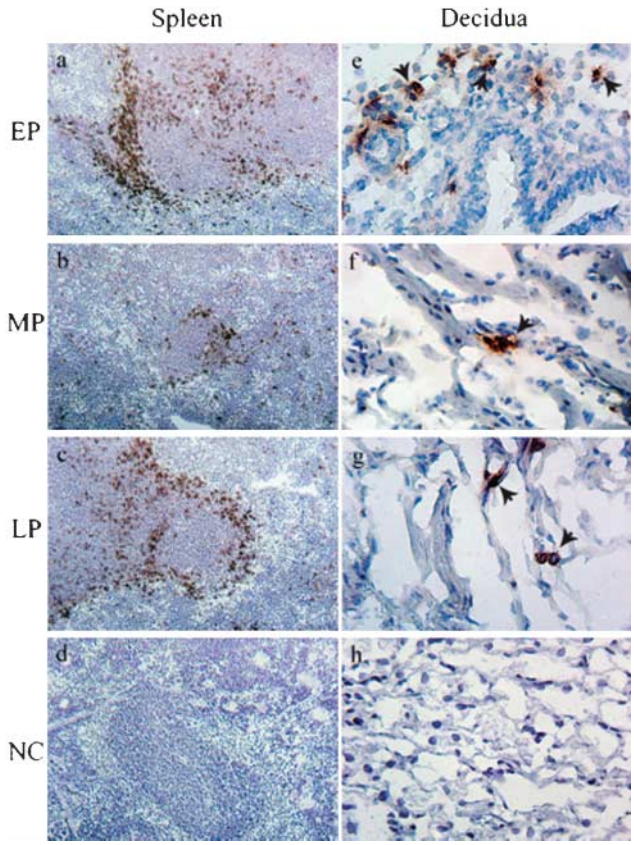

Figure 1 Immunostaining of dendritic cells in spleen and decidua of pregnant Balb/c mice. Cryosections were prepared from spleen (a–d) and uterus (e–h) of female mice and stained with CD11c. d and h are negative control slides in which primary antibody was substituted by species-matched control sera. (a–d, 100×; e–h, 200×). Black arrows show decidual dendritic cells. NC, negative control; EP, early pregnancy; MP, mid-pregnancy; LP, late pregnancy.

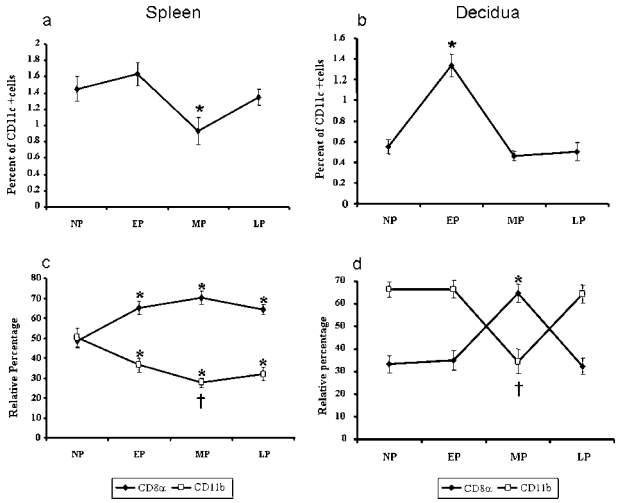

Figure 2 Comparative analysis of DCs ($CD11c^+$), LDCs ($CD11c^+$ $CD8\alpha^+$), and MDCs ($CD11c^+$ $CD11b^+$) in spleen and decidua of pregnant Balb/c mice. Non-pregnant mice served as controls. Cryosections were prepared from spleen and uterus of female mice and stained with CD11c (a and b). Positive cells were counted and expressed as a percentage of the total number of nucleated cells. (a) Frequency of splenic DCs declined to its lowest level during mid-pregnancy when compared with early and late pregnancy periods or non-pregnant mice (*$P<0.01$). (b) At early pregnancy, frequency of decidual DCs was highest relative to other stages or non-pregnant mice (*$P<0.01$). For immunophenotyping, double color immunohistochemistry staining was carried out using CD11c and one of the CD8α or CD11b antibodies. Double positive cells were counted and expressed as a percentage of the total number of $CD11c^+$ cells. (c) At all periods of pregnancy, relative percentage of splenic LDCs was significantly increased, while those of MDCs decreased when compared with non-pregnant mice (*$P<0.05$). Also, the relative percentage of myeloid DCs was significantly lower at mid-gestation when compared with early or late pregnancy ($^{\dagger}P<0.05$). (d) The middle stage of pregnancy was a critical point in that relative percentages of decidual LDCs and MDCs were reversed when compared with other stages of pregnancy (*,$^{\dagger}P<0.01$). NP, non-pregnant; EP, early pregnancy; MP, mid-pregnancy; LP, late pregnancy.

CD11c, MHC-II, CD8α, DEC- 205, CD11b, and CD86 at each stage of pregnancy.

Dendritic cells in spleen of pregnant mice

DCs were present throughout the gestation in spleen of pregnant mice (Fig. 1a–c), but their percentage was highest at early pregnancy and lowest at mid-gestation ($P<0.01$; Fig. 2a). DCs were heavily distributed around the lymphoid follicles of the white pulps with some scattered in the red pulp (Fig. 1a). The average density of splenic DCs at early, middle, and late pregnancy was 1.63 ± 0.14, 0.93 ± 0.17, and $1.35\pm0.1\%$ respectively (Fig. 2a). In all stages studied, no statistical difference was found in relative percentage of lymphoid ($CD11c^+$ $CD8\alpha^+$) DCs, although the relative percentage of MDCs was significantly lower at mid-gestation when compared with early or late pregnancy ($P<0.05$; Fig. 2c). On an average, 65.2 ± 3.4, 70.2 ± 3.5, and $64.2\pm3.3\%$ of splenic DCs expressed CD8α at early, middle, and late pregnancy respectively, while the relative percentage of $CD11b^+$ DCs at the above stages was 36.5 ± 4.7, 27.6 ± 3.6, and $31.9\pm2.4\%$. At all periods of pregnancy, the relative percentage of splenic LDCs was significantly increased, while those of MDCs decreased when compared with non-pregnant mice ($P<0.05$; Fig. 2c).

Our results also showed that the ratio of LDC/MDC at mid-gestation in spleens of pregnant mice was significantly higher than those of other stages ($P<0.05$; data not shown). In addition, we found that the majority of the splenic DCs express MHC-II (97.9 ± 1.8) and CD86 (91.5 ± 5.5) (Table 2). The expression of DEC-205 on DCs correlated strictly with that of CD8α ($r=0.87$; Fig. 3). No differences were found between different stages of pregnancy with regard to expression of CD86 and MHC-II on splenic DCs.

Dendritic cells in decidua of pregnant mice:

DCs were present in decidua at all stages of pregnancy (Fig. 1e–g). The comparative analysis of decidual DCs

Table 2 Expression of MHC-II and CD86 on splenic and decidual CD11c$^+$ dendritic cells during different periods of pregnancy.

	Spleen				Decidua			
	NP	EP	MP	LP	NP	EP	MP	LP
MHC-II	97.3±2.2	98.2±1.7	98±1.9	97±1.6	98±1.5	98.6±1.4	99±0.6	97.8±1.9
CD86	85.3±6.2	92.3±4.8	88±5.1	94.2±6.7	87.9±5.4	91±2.8	93±4.7	95.1±5.6

NP, non-pregnant; EP, early pregnancy; MP, mid-pregnancy; LP, late pregnancy. Non-pregnant mice served as control.

among three stages of pregnancy and non-pregnant mice showed that the average density of decidual DCs was significantly higher at early pregnancy ($P<0.01$; Fig. 2b). The average density of DCs was 1.34±0.11, 0.46±0.05, and 0.5±0.09% at early, middle, and late pregnancy respectively. Interestingly, the average densities of splenic and decidual DCs followed similar variations at different stages of pregnancy. We examined the distribution of decidual DCs at various stages of pregnancy. DCs were present at all areas of uterus, including myometrium, mesometrium, stroma, and subepithelial layers. At early pregnancy, there were numerous DCs in decidua concentrated subadjacent to the luminal epithelial layers (Fig. 1e), whereas at middle or late pregnancy DCs were randomly distributed in stroma and around the glandular and luminal epithelial layers.

The relative percentage of LDCs was significantly higher at mid-gestation when compared with early or late pregnancy and non-pregnant mice ($P<0.01$; Fig. 2d). We found that 35.1±4.3, 64.6±4, and 32.3±3.6% of decidual DCs expressed CD8α at early, middle, and late pregnancy respectively. Inversely, the relative percentage of decidual MDCs at middle pregnancy was significantly lower in comparison with other stages of pregnancy or non-pregnant mice ($P<0.01$; Fig. 2d). The relative percentage of MDC at early, middle, and late pregnancy was 64.6±3.9, 34.4±5.5, and 64.3±4% respectively.

As expected, the LDC/MDC ratio was higher at mid-gestation when compared with other stages ($P<0.01$; data not shown). There was no statistical difference, with regard to MHC-II or CD86 expression on decidual DCs (Table 2), between various stages of pregnancy and the majority of decidual DCs expressed these two markers. Immunostaining with DEC-205 showed that as for CD8α, DEC-205$^+$ CD11c$^+$ DCs were present in greater numbers at mid-gestation ($P<0.01$).

Discussion

In this study, we examined the relative percentage, localization, and immunophenotype (with emphasis on myeloid and lymphoid markers) of decidual and splenic DCs of different stages of mouse pregnancy. Analysis of DC frequency in spleen of pregnant mice revealed that on an average, DCs comprised 1.3±0.11% of all splenic nucleated cells, which was statistically similar to what we found in non-pregnant mice (1.45±0.15%) (Zarnani *et al.* 2006). The average density of DCs was statistically lower at mid-gestation when compared with other stages of pregnancy or non-pregnant mice. We did not find any report on splenic DCs during gestation. Published data on peripheral DCs of pregnant women on different stages of pregnancy, however, showed that the number of peripheral blood DCs was significantly lower in the second trimester, when compared with the first and third trimesters of normal pregnancy (Rmochwal-Kolarz *et al.* 2003*a*, 2003*b*, Yoshimura *et al.* 2003). The reason for this phenomenon is not clarified, but Kolarz *et al.* (Rmochwal-Kolarz *et al.* 2003*b*) proposed that lower frequency of blood DCs at mid-gestation is due to their migration into the uterus. Our findings revealed that, in parallel to splenic DCs, the frequency of decidual DCs was also decreased at mid-gestation and therefore it does not seem that such migration is responsible for this phenomenon. It is possible that DCs are hormonally controlled during normal pregnancy. In rats, progesterone concentration reaches the highest level at mid-gestation (Varga *et al.* 1981) and a decrease in DC frequency during this period may be associated with the change in progesterone concentration.

Having considered that the relative frequency of splenic LDCs increased (although this was not statistically significant), while those of MDCs decreased significantly at mid-gestation, it seems that reduction in splenic DC frequency during this period is mainly due to a decrease in MDC populations.

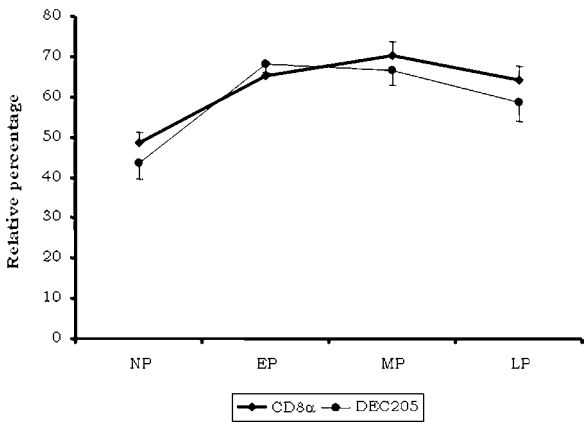

Figure 3 Expression of DEC-205 and CD8α on splenic CD11c$^+$ dendritic cells during different periods of pregnancy. Non-pregnant mice served as controls. As shown, there is considerable correlation between the expressions of these markers on dendritic cells. NP, non-pregnant; EP, early pregnancy; MP, mid-pregnancy; LP, late pregnancy.

It has recently been reported that the percentage of LDCs and the LDC/MDC ratio were significantly lower in the second trimester, when compared with the first or third trimesters of normal human pregnancy (Rmochwal-Kolarz *et al.* 2003*b*, Yoshimura *et al.* 2003). This discrepancy can be explained by considering the opposite functions of DC subsets, in human and in mice. In human, myeloid and lymphoid DCs promote Th1 and Th2 immunities respectively, while in mice, MDCs are proved to induce a Th2-biased response and LDCs have the ability to induce a Th1-biased cytokine response (Banchereau *et al.* 2000, Moser & Murphy 2000). Furthermore, in contrast to non-pregnant mice in which splenic myeloid and lymphoid DCs were present equally (48.6 ± 2.7 vs $50.3 \pm 3.4\%$), lymphoid DCs were more prominent is spleens of pregnant mice (66.5 ± 3.4 vs $32 \pm 3.6\%$). We did not find statistical differences in MHC-II and CD86 expression on splenic or decidual DCs between various stages of pregnancy. However, regarding the fact that we did not analyze quantitative expression of these markers on DCs, the existence of such differences could not be ruled out. Kammerer *et al.* (2000) reported for the first time that $CD83^+$ mature DCs were present in the human decidua (mean density $= 4.97 \pm 1.88/mm^2$). They later traced first-trimester decidual DCs using anti-DC-SIGN antibody and showed that the frequency of DC-$SIGN^+$ DC was $37 \pm 23/mm^2$, which was obviously higher than the frequently of $CD83^+$ mature DCs (Kammerer *et al.* 2003). Taking all these data into account, it seems that in their first report, Kammerer *et al.* underestimated the frequency of decidual DCs as the majority of these cells were immature and CD83 is expressed exclusively on mature DCs. Indeed, decidua is a non-lymphoid tissue and DCs resident in such tissues are generally immature, undergoing maturation only after antigen uptake and migration to regional lymph nodes (Banchereau *et al.* 2000).

It was recently reported that the frequency of murine endometrial DCs was highest at estrus, a phase in estrous cycle in which mating occurs (Zarnani *et al.* in press). In the present study, we observed that mating was associated with further recruitment of large numbers of DCs into the early decidua. One important explanation for this phenomenon may be the uptake and processing of seminal proteins by DCs, leading to the induction of appropriate immune response to paternal antigens. It seems that DC recruitment to decidua is a feature of inflammatory responses induced by insemination. Introduction of the male ejaculate has been shown to cause immediate changes in leukocyte populations in the female reproductive tract of several species, including rodents, pigs, rabbits, and humans (Lovell & Getty 1968, Phillips & Mahler 1977, Pandya & Cohen 1985, De *et al.* 1991, Robertson & Sharkey 2001). Within hours of mating in mice, specific factors in seminal plasma including transforming growth factor β (TGF-β) originating in the seminal vesicles, target epithelial cells lining the uterine lumen to elicit a surge in release of proinflammatory cytokines and chemokines, such as granulocyte-monocyte colony stimulating factor (GM-CSF), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), which in turn provoke an influx and activation of macrophages, DCs, and neutrophils in a response resembling a clinical inflammatory reaction (Robertson *et al.* 1996*a*, Tremellen *et al.* 1998). According to Robertson *et al.* GM-CSF receptor is expressed on the majority of endometrial immune cells, including macrophages, DCs, and neutrophils (Robertson *et al.* 1992, 1996*a*).

Interestingly, it has been shown that intradermal injection of recombinant GM-CSF in patients with leprosy (Kaplan *et al.* 1992) or in mice (O'Sullivan *et al.* 1996) results in recruitment of langerhans cells in the skin. In mice, GM-CSF mRNA expression and protein secretion are upregulated 20-fold after insemination (Robertson *et al.* 1992). It is notable that GM-CSF mRNA expression remains elevated for 48 h and then declines as progesterone levels increase on day 3 and day 4 of pregnancy (Robertson *et al.* 1996*a*).

There is no direct evidence to show that progesterone may play a role in DC recruitment to the female reproductive tract, but it seems that this hormone may inhibit DC recruitment through downregulation of GM-CSF production. Pretreatment of ovariectomized mice with estradiol, but not progesterone, resulted in the induction of GM-CSF production and estradiol-induced increase was inhibited by the co-administration of progesterone (Robertson *et al.* 1996*b*). As pregnancy proceeds, the progesterone level increases and thus it is conceivable that the frequency of decidual DCs declines with the progression of pregnancy. This hypothesis is in line with our findings. Indeed, in rats, progesterone levels peak at mid-gestation (Varga *et al.* 1981) when, according to our results, the frequency of splenic or decidual DCs declines to its lowest level.

Comparing the relative frequency of decidual lymphoid and myeloid DCs, we found that except for the mid-gestation, MDCs predominate. In mice, MDCs shift T cell responses toward Th2 immunity and considering that the Th2 response is necessary for a successful pregnancy, our findings can be explained. Predominancy of the LDC subset (which promotes Th1 immunity) at mid-gestation, however, seems to be contrary to the established Th2 paradigm of pregnancy.

In general, LDCs induce a limited $CD4^+$T cell response that is associated with marked T cell apoptosis and induction of tolerance (Inaba *et al.* 1997, Vandenabeele & Wu 1999, Banchereau *et al.* 2000). Therefore, it may be suggested that the predominancy of decidual LDCs at mid-gestation may serve as an important role in controlling destructive responses of maternal immune system against paternal antigens through induction of immunological tolerance. Our findings also propounded another explication for this phenomenon. We found that treatment of DCs with decidual supernatant obtained

from allogenic pregnant mice at mid-gestation markedly blocked their ability to induce antigen-specific IFNγ production by cultured lymph node cells (A H Zarnani, unpublished observations). Thus, this Th2 promoting effect of uterine microenvironment could compensate for inherent Th1-skewing capacity of LDCs. There are plenty of reports indicating that the main functional properties of DCs, i.e., antigen presentation and Th1/Th2 induction capacity, are controlled by environmental instructions (Stumbles *et al.* 1998, Kapsenberg *et al.* 1999, Liu *et al.* 2002, Vlad *et al.* 2003) and that the same subset of DCs in different tissues is capable of inducing distinct immune responses (Iwasaki & Kelsall 1999). These explanations might be true for predominancy of LDCs in the spleens of pregnant mice.

In a recent study, Blois *et al.* (2004*a*) found lower frequency of splenic and uterine $CD11c^+$ DCs at early pregnancy and that frequency of such cells reached its highest level at mid-gestation. In addition, in blood, the percentage of LDCs was significantly highest at mid-gestation, while uterine LDCs predominated at early pregnancy. What we found in the present study is in contrast to the above-mentioned findings. Although both studies have monitored phenotype and lineage of DCs in different periods of murine pregnancy, however, there are some fundamental differences. First, in their study, Blois *et al.* used CBA/J×DBA/2 model, which is known to be abortion prone and according to the several reports may suffer from many immunological defects leading to abortion, including imbalance of DC population, as the same group (Blois *et al.* 2004*b*) reported in their next study that therapy with DCs normalize abortion rate in this model. Indeed, in the CBA/J×DBA/2 model, fetal resorption occurs at mid-gestation, a critical period that according to Blois *et al.* and also our findings during which major changes are happening, and it is interesting to note that the major differences between findings of Blois *et al.* and the present study are in this period. Moreover, Rmochwal-Kolarz (2003*a*) also reported lower frequency of peripheral blood DCs in the second trimester of normal human pregnancy supporting the data presented here. On the other hand, according to Blois *et al.*, the percentage of the uterine $CD11c^+$ cells at mid-gestation is about 15%, which is unexpectedly high, as the frequency of these cells in such lymphoid organs as spleen, which is heavily populated by DCs is around 1.5% (Steinman & Cohn 1973, Zarnani *et al.* 2006). Second, we analyzed decidual and splenic DCs instead of looking at uterine and peripheral blood DCs, which might be different in terms of DC frequency or phenotype. Third, completely different methods (immunohistochemistry versus flow cytometry) have been used for immunophenotyping of DCs in these studies.

Differential recruitment of MDCs and LDCs to the feto-maternal interface at different stages of pregnancy is probably influenced by different arrays of chemokines produced *in situ* (Kayisli *et al.* 2002, Penna *et al.* 2002). Conversely, different subsets of DCs could modulate the outgoing immune response through production of different chemokines, which are able to recruit Th1/Th2/Treg cells (Bonecchi *et al.* 1998, Loetscher *et al.* 1998, Iellem *et al.* 2001, Sebastiani *et al.* 2001).

In conclusion, we characterized distribution and immunophenotype, with emphasis on myeloid and lymphoid markers, of $CD11c^+$ DCs in the decidua and spleen of pregnant mice during different stages of gestation. Data presented here, reveal that frequency and subset of splenic and decidual DCs are finely tuned in a timely manner throughout pregnancy. Therefore, such balance may be relevant to the immunology of successful pregnancy and shed light on the complex networks of immunoregulation at the feto-maternal interface.

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